IN THE SPECIFICATION:

Please replace the specification with the Substitute Specification beginning on the next page and numbering pages 1-57.

Description

~~A GENE SWITCH~~

METHOD FOR CONTROLLING GENE EXPRESSION IN A CELL

Cross Reference to Related Applications

This is a divisional of co-pending U.S. Patent Application Serial No. 09/564,418, filed May 3, 2000, which is a divisional of U.S. Patent Application Serial No. 08/653,648, filed May 24, 1996, now U.S. Patent No. 6,379,945. The entire contents of U.S. Patent Application Serial Nos. 08/653,648 and 09/564,418 are herein incorporated by reference.

Technical Field

The present invention relates to the identification and characterisation of insect steroid receptors from the Lepidoptera species *Heliothis virescens*, and the nucleic acid encoding therefor. The present invention also relates to the use of such receptors, and such nucleic acid, particularly, but not exclusively, in screening methods, and gene switches. By gene switch we mean a gene sequence which is responsive to an applied exogenous chemical inducer enabling external control of expression of the gene controlled by said gene sequence.

Background Art

Lipophilic hormones such as steroids induce changes in gene expression to elicit profound effects on growth, cellular differentiation, and homeostasis. These hormones recognise intracellular receptors that share a common modular structure consisting of three main functional domains: a variable amino terminal region that contains a transactivation domain, a DNA binding domain, and a ligand binding domain on the carboxyl side of the molecule. The DNA binding domain contains nine invariant cysteines, eight of which are involved in zinc coordination to form a two-finger structure. In the nucleus the hormone-receptor complex binds to specific enhancer-like sequences called hormone response elements (HREs) to modulate transcription of target genes.

The field of insect steroid research has undergone a revolution in the last three years as a result of the cloning and preliminary characterisation of the first steroid receptor member genes. These developments suggest the time is ripe to try to use this knowledge

to improve our tools in the constant fight against insect pests. Most of the research carried out on the molecular biology of the steroid receptor superfamily has been on *Drosophila melanogaster* (Diptera), see for example International Patent Publication No. WO 91/13167, with some in *Manduca* and *Galleria* (Lepidoptera).

It has been three decades since 20-hydroxyecdysone was first isolated and shown to be involved in the regulation of development of insects. Since then work has been carried out to try to understand the pathway by which this small hydrophobic molecule regulates a number of activities. By the early 1970s, through the studies of Clever and Ashburner, it was clear that at least in the salivary glands of third instar *Drosophila* larvae, the application of ecdysone lead to the reproducible activation of over a hundred genes. The ecdysone receptor in this pathway is involved in the regulation of two classes of genes: a small class (early genes) which are induced by the ecdysone receptor and a large class (late genes) which are repressed by the ecdysone receptor. The early class of genes are thought to have two functions reciprocal to those of the ecdysone receptor; the repression of the early transcripts and the induction of late gene transcription. Members of the early genes so far isolated and characterised belong to the class of molecules with characteristics similar to known transcription factors. They are thus predicted to behave as expected by the model of ecdysone action (Ashburner, 1991). More recently, the early genes E74 and E75 have been shown to bind both types of ecdysone inducible genes (Thummel et al., 1990; Segraves and Hogness, 1991), thus supporting their proposed dual activities. It should be noted however, that the activation of a hierarchy of genes is not limited to third instar larvae salivary glands, but that the response to the ecdysone peak at the end of larval life is observed in many other tissues, such as the imaginal disks (i.e. those tissues which metamorphose to adult structures) and other larval tissues which histolyse at the end of larval life (e.g. larval fat body). The model for ecdysone action as deduced by studying the third instar chromosome puffing may not apply to the activation of ecdysone regulated genes in adults. In other words, the requirement for other factors in addition to the active ecdysone receptor must be satisfied for correct developmental expression (e.g. the *Drosophila* yolk protein gene expression in adults is under control of doublesex, the last gene in the sex determination gene hierarchy).

The ecdysone receptor and the early gene E75 belong to the steroid receptor superfamily. Other *Drosophila* genes, including ultraspiracle, tailless, sevenup and FTZ-FI, also belong to this family. However, of all these genes only the ecdysone receptor is

known to have a ligand, and thus the others are known as orphan receptors. Interestingly, despite the ultraspiracle protein ligand binding region sharing 49% identity with the vertebrate retinoic X receptor (RXR) ligand binding region (Oro et al., 1990), they do not share the same ligand (i.e. the RXR ligand is 9-cis retinoic acid) (Heymann et al., 1992 and Mangelsdorf et al., 1992). All the *Drosophila* genes mentioned are involved in development, ultraspiracle for example, is required for embryonic and larval abdominal development. The protein products of these genes all fit the main features of the steroid receptor superfamily (Evans, 1988; Green and Chambon, 1988, Beato, 1989): i.e., they have a variable N terminus region involved in ligand independent transactivation (Domains A and B), a highly conserved 66-68 amino acid region which is responsible for the binding of DNA at specific sites (Domain C), a hinge region thought to contain a nuclear translocation signal (Domain D), and a well conserved region containing the ligand binding region, transactivation sequences and the dimerisation phase (Domain E). The last region, domain F, is also very variable and its function is unknown.

Steroid receptor action has been elucidated in considerable detail in vertebrate systems at both the cellular and molecular levels. In the absence of ligand, the receptor molecule resides in the cytoplasm where it is bound by Hsp90, Hsp70, and p59 to form the inactive complex (Evans, 1988). Upon binding of the ligand molecule by the receptor a conformational change takes place which releases the Hsp90, Hsp70 and p59 molecules, while exposing the nuclear translocation signals in the receptor. The ligand dependent conformational change is seen in the ligand binding domain of both progesterone and retinoic acid receptors (Allan et al., 1992a). This conformational change has been further characterised in the progesterone receptor and was found to be indispensable for gene transactivation (Allan et al., 1992b). Once inside the nucleus the receptor dimer binds to the receptor responsive element at a specific site on the DNA resulting in the activation or repression of a target gene. The receptor responsive elements usually consist of degenerate direct repeats, with a spacer between 1 and 5 nucleotides, which are bound by a receptor dimer through the DNA binding region (Domain C).

Whereas some steroid hormone receptors are active as homodimers others act as heterodimers. For example, in vertebrates, the retinoic acid receptor (RAR) forms heterodimers with the retinoic X receptor (RXR). RXR can also form heterodimers with the thyroid receptor, vitamin D receptor (Yu et al., 1991; Leid et al., 1992) and peroxisome activator receptor (Kliewer et al., 1992). Functionally the main difference

between homodimers and heterodimers is increased specificity of binding to specific response elements. This indicates that different pathways can be linked, co-ordinated and modulated, and more importantly this observation begins to explain the molecular basis of the pleotropic activity of retinoic acid in vertebrate development (Leid et al., 1992b). Similarly, the *Drosophila* ultraspiracle gene product was recently shown to be capable of forming heterodimers with retinoic acid, thyroid, vitamin D and peroxisome activator receptors and to stimulate the binding of these receptors to their target responsive elements (Yao et al., 1993). More significantly, the ultraspiracle gene product has also been shown to form heterodimers with the ecdysone receptor, resulting in cooperative binding to the ecdysone response element and capable of rendering mammalian cells ecdysone responsive (Yao et al., 1992). The latter is of importance since transactivation of the ecdysone gene alone in mammalian cells fails to elicit an ecdysone response (Koelle et al., 1991), therefore suggesting that the ultraspiracle gene product is an integral component of a functional ecdysone receptor (Yao et al., 1992). It is possible that the ultraspiracle product competes with other steroid receptors or factors to form heterodimers with the ecdysone receptor. Moreover it remains to be investigated if ultraspiracle is expressed in all tissues of the *Drosophila* larvae. Despite ultraspiracle being necessary to produce a functional ecdysone receptor, the mechanism by which this activation takes place is as yet undetermined.

Summary

We have now isolated and characterised the ecdysone steroid receptor from *Heliothis virescens* (hereinafter HEcR). We have found that surprisingly unlike the *Drosophila* ecdysone steroid receptor (hereinafter DEcR), in reports to-date, HEcR can be induced by known non-steroidal inducers. It will be appreciated that this provides many advantages for the system.

Steroids are difficult and expensive to make. In addition, the use of a non-steroid as the inducer allows the system to be used in agrochemical and pharmaceutical applications, not least because it avoids application of a steroid which is already present in insects and/or mammals. For example, it would not be feasible to use a gene switch in a mammalian cell which was induced by a naturally occurring steroidal inducer. It will also

be appreciated that for environmental reasons it is advantageous to avoid the use of steroids as inducers.

According to one aspect of the present invention there is provided DNA having the sequence shown in ~~Seq ID No. 2~~ SEQ ID NO: 2, wherein ~~Seq ID No 2~~ **SEQ ID NO: 2** gives the sequence for the HEcR.

According to another aspect of the present invention there is provided DNA having part of the sequence shown in ~~Seq ID No. 2~~ **SEQ ID NO: 2**, which encodes for the HEcR ligand binding domain.

According to another aspect of the present invention there is provided DNA having part of the sequence shown in ~~Seq ID No. 2~~ **SEQ ID NO: 2**, which encodes for the HEcR DNA binding domain.

According to yet another aspect of the present invention there is provided DNA having part of the sequence shown in ~~Seq ID No. 2~~ **SEQ ID NO: 2**, which encodes for the HEcR transactivation domain.

According to a further aspect of the present invention there is provided DNA having part of the sequence shown in ~~Seq ID No. 2~~ **SEQ ID NO: 2**, which encodes for the HEcR hinge domain.

According to a still further aspect of the present invention there is provided DNA having part of the sequence shown in ~~Seq ID No. 2~~ **SEQ ID NO: 2**, which encodes for the HEcR carboxy terminal region.

According to one aspect of the present invention there is provided DNA having the sequence shown in ~~Seq ID No. 3~~ **SEQ ID NO: 3**, wherein ~~Seq ID No 3~~ **SEQ ID NO: 3** gives the sequence for the HEcR.

According to another aspect of the present invention there is provided DNA having part of the sequence shown in ~~Seq ID No. 3~~ **SEQ ID NO: 3**, which encodes for the HEcR ligand binding domain.

According to another aspect of the present invention there is provided DNA having part of the sequence shown in ~~Seq ID No. 3~~ **SEQ ID NO: 3**, which encodes for the HEcR DNA binding domain.

According to yet another aspect of the present invention there is provided DNA having part of the sequence shown in ~~Seq ID No. 3~~ **SEQ ID NO: 3**, which encodes for the HEcR transactivation domain.

According to a further aspect of the present invention there is provided DNA having part of the sequence shown in ~~Seq ID No. 3~~ **SEQ ID NO: 3**, which encodes for the HEcR hinge domain.

According to a still further aspect of the present invention there is provided DNA having part of the sequence shown in ~~Seq ID No. 3~~ **SEQ ID NO: 3**, which encodes for the HEcR carboxy terminal region.

According to one aspect of the present invention there is provided DNA having the sequence shown in ~~Seq ID No. 4~~ **SEQ ID NO: 4**, wherein ~~Seq ID No 4~~ **SEQ ID NO: 4** gives the sequence for the HEcR.

According to another aspect of the present invention there is provided DNA having part of the sequence shown in ~~Seq ID No. 4~~ **SEQ ID NO: 4**, which encodes for the HEcR ligand binding domain.

According to another aspect of the present invention there is provided DNA having part of the sequence shown in ~~Seq ID No. 4~~ **SEQ ID NO: 4**, which encodes for the HEcR DNA binding domain.

According to yet another aspect of the present invention there is provided DNA having part of the sequence shown in ~~Seq ID No. 4~~ **SEQ ID NO: 4**, which encodes for the HEcR transactivation domain.

According to a further aspect of the present invention there is provided DNA having part of the sequence shown in ~~Seq ID No. 4~~ **SEQ ID NO: 4**, which encodes for the HEcR hinge domain.

According to a still further aspect of the present invention there is provided DNA having part of the sequence shown in ~~Seq ID No. 4~~ **SEQ ID NO: 4**, which encodes for the HEcR carboxy terminal region.

Detailed Description

As mentioned above, steroid receptors are eukaryotic transcriptional regulatory factors which, in response to the binding of the steroid hormone, are believed to bind to specific DNA elements and activate transcription. The steroid receptor can be divided into six regions, designated A to F, using alignment techniques based on shared homology with other members of the steroid hormone receptor superfamily. Krust et al. **(1986)** identified two main regions in the receptor, C and E. Region C is hydrophilic and is unusual in its high content in cysteine, lysine and arginine. It corresponds to a DNA-binding domain,

sometimes referred to as the "zinc finger". It is the DNA binding domain which binds to the upstream DNA of the responsive gene. Such upstream DNA is known as the hormone response element or HRE for short. Region E is hydrophobic and is identified as the hormone (or ligand) binding domain. Region E can be further subdivided into regions E1, E2 and E3.

The region D, which separates domains C and E is highly hydrophobic and is flexible. It is believe that communication between domains E and C involves direct contact between them through region D, which provides a hinge between the two domains. Region D is therefore referred to as the hinge domain.

The mechanism of the receptor appears to require it to interact with some element(s) of the transcription machinery over and above its interactions with the hormone and the hormone response element. N-terminal regions A and B perform such a function and are jointly known as the transactivation domain. The carboxy terminal region is designated F.

The domain boundaries of the HEcR can be defined as follows:

DOMAIN	**INTERVALS**	
	base pairs	**amino acids**
Transactivating (A/B)	114-600	1-162
DNA Binding (C)	601-798	163-228
Hinge (D)	799-1091	229-326
Ligand Binding (E)	1092-1757	327-545
C-Terminal End (F)	1758-1844	546-577

The DNA binding domain is very well defined and is 66 amino acids long, thus providing good boundaries. The above intervals have been defined using the multiple alignment for the ecdysone receptors (Figure 5).

The present invention also includes DNA which shows homology to the sequences of the present invention. Typically homology is shown when 60% or more of the ~~nucletides~~ **nucleotides** are common, more typically 65%, preferably 70%, more preferably 75%, even more preferably 80% or 85%, especially preferred are 90%, 95%, 98% or 99% or more homology.

The present invention also includes DNA which hybridises to the DNA of the present invention and which codes for at least part of the *Heliothis* ecdysone receptor transactivation domain, DNA binding domain, hinge domain, ligand binding domain and/or carboxy terminal region. Preferably such hybridisation occurs at, or between, low and high stringency conditions. In general terms, low stringency conditions can be defined as 3X ~~x SCC~~ **SSC** at about ambient temperature to about 65°C, and high stringency conditions as 0.1X x SSC at about 65°C. SSC is the name of a buffer of 0.15M NaCl, 0.015M trisodium citrate. 3X x SSC is three times as strong as SSC and so on.

The present invention further includes DNA which is degenerate as a result of the genetic code to the DNA of the present invention and which codes for a polypeptide which is at least part of the *Heliothis* ecdysone receptor transactivation domain, DNA binding domain, hinge domain, ligand binding domain and/or carboxy terminal region.

The DNA of the present invention may be cDNA or DNA which is in an isolated form.

According to another aspect of the present invention there is provided a polypeptide comprising the *Heliothis* ecdysone receptor or a fragment thereof, wherein said polypeptide is substantially free from other proteins with which it is ordinarily associated, and which is coded for by any of the DNA of the present invention.

According to another aspect of the present invention there is provided a polypeptide which has the amino acid sequence of ~~Seq ID No. 4~~ **SEQ ID NO: 5** or any allelic variant or derivative thereof, wherein ~~Seq ID No. 4~~ **SEQ ID NO: 5** gives the amino acid sequence of the HEcR polypeptide.

According to another aspect of the present invention there is provided a polypeptide which has part of the amino acid sequence of ~~Seq ID No. 4~~ **SEQ ID NO: 5** or any allelic variant or derivative thereof, which sequence provides the HEcR ligand binding domain.

According to another aspect of the present invention there is provided a polypeptide which has part of the amino acid sequence of ~~Seq ID No. 4~~ **SEQ ID NO: 5** or any allelic variant or derivative thereof, which sequence provides the HEcR DNA binding domain.

According to yet another aspect of the present invention there is provided a polypeptide which has part of the amino acid sequence of ~~Seq ID No. 4~~ **SEQ ID NO: 5** or

any allelic variant or derivative thereof, which sequence provides the HEcR transactivation domain.

According to a further aspect of the present invention there is provided a polypeptide which has the amino acid sequence of a part of ~~Seq ID No. 4~~ **SEQ ID NO: 5** or any allelic variant or derivative thereof, which sequence provides the HEcR hinge domain.

According to a still further aspect of the present invention there is provided a polypeptide which has the amino acid sequence of a part of ~~Seq ID No. 4~~ **SEQ ID NO: 5** or any allelic variant or derivative thereof, which sequence provides the HEcR carboxy terminal region.

For the avoidance of doubt, spliced variants of the amino acid sequences of the present invention are included in the present invention.

Preferably, said derivative is a homologous variant which has conservative amino acid changes. By conservation amino acid changes we mean replacing an amino acid from one of the amino acid groups, namely hydrophobic, polar, acidic or basic, with an amino acid from within the same group. An ~~examples~~ **example** of such a change is the replacement of valine by methionine and vice versa.

According to another aspect of the present invention there is provided a fusion polypeptide comprising at least one of the polypeptides of the present invention functionally linked to an appropriate non-*Heliothis* ecdysone receptor domain(s).

According to an especially preferred embodiment of the present invention the HEcR ligand binding domain of the present invention is fused to a DNA binding domain and a transactivation domain.

According to another embodiment of the present invention the DNA binding domain is fused to a ligand binding domain and a transactivation domain.

According to yet another embodiment of the present invention the transactivation domain is fused to a ligand binding domain and a DNA binding domain.

The present invention also provides recombinant DNA encoding for these fused polypeptides.

According to an especially preferred embodiment of the present invention there is provided recombinant nucleic acid comprising a DNA sequence encoding the HEcR ligand binding domain functionally linked to DNA encoding the DNA binding domain and transactivation domain from a glucocorticoid receptor.

According to yet another aspect of the present invention there is provided recombinant nucleic acid comprising a DNA sequence comprising a reporter gene operably linked to a promoter sequence and a hormone response element which hormone response element is responsive to the DNA bonding domain encoded by the DNA ~~of~~ of the present invention.

According to another aspect of the present invention there is provided a construct transformed with nucleic acid, recombinant DNA, a polypeptide or a fusion polypeptide of the present invention. Such constructs include plasmids and phages suitable for transforming a cell of interest. Such constructs will be well known to those skilled in the art.

According to another aspect of the present invention there is provided a cell transformed with nucleic acid, recombinant DNA, a polypeptide, or a fusion polypeptide of the present invention.

Preferably the cell is a plant, fungus or mammalian cell.

For the avoidance of doubt fungus includes yeast.

The present invention therefore provides a gene switch which is operably linked to a foreign gene or a series of foreign genes whereby expression of said foreign gene or said series of foreign genes may be controlled by application of an effective exogenous inducer.

Analogs of ecdysone, such as Muristerone A, are found in plants and disrupt the development of insects. It is therefore proposed that the receptor of the present invention can be used be in plants transformed therewith as an insect control mechanism. The production of the insect-damaging product being controlled by an exogenous inducer. The insect-damaging product can be ecdysone or another suitable protein.

The first non-steroidal ecdysteroid agonists, dibenzoyl hydrazines, typified by RH-5849 [1,2-dibenzoyl, 1-tert-butyl hydrazide], which is commercially available as an insecticide from Rohm and Haas, were described back in 1988. Another commercially available compound in this series is RH-5992 [tebufenozide, 3,5-dimethylbenzoic acid 1-1 (1,1-dimethylethyl)-2(4-ethylbenzoyl) hydrazide]. These compounds mimic 20-hydroxyecdysone (20E) in both *Manduca sexta* and *Drosophila melanogaster*. These compounds have the advantage that they have the potential to control insects using ecdysteroid agonists which are non-steroidal. Further Examples of such dibenzoyl hydrazines are given in U.S. Patent No. 5,117,057 to Rohm and Haas, and Oikawa et al.,

Pestic Sci, 41, 139-148 (1994). However, it will be appreciated that any inducer of the gene switch of the present invention, whether steroidal or non-steroidal, and which is currently or becomes available, may be used.

The gene switch of the present invention, then, when linked to an exogenous or foreign gene and introduced into a plant by transformation, provides a means for the external regulation of expression of that foreign gene. The method employed for transformation of the plant cells is not especially germane to this invention and any method suitable for the target plant may be employed. Transgenic plants are obtained by regeneration from the transformed cells. Numerous transformation procedures are known from the literature such as agroinfection using *Agrobacterium tumefaciens* or its Ti plasmid, electroporation, microinjection or plants cells and protoplasts, microprojectile transformation, to mention but a few. Reference may be made to the literature for full details of the known methods.

Neither is the plant species into which the chemically inducible sequence is inserted particularly germane to the invention. Dicotyledonous and monocotyledonous plants can be transformed. This invention may be applied to any plant for which transformation techniques are, or become, available. The present invention can therefore be used to control gene expression in a variety of genetically modified plants, including field crops such as canola, sunflower, tobacco, sugarbeet, and cotton; cereals such as wheat, barley, rice, maize, and sorghum; fruit such as tomatoes, mangoes, peaches, apples, pears, strawberries, bananas and melons; and vegetables such as carrot, lettuce, cabbage and onion. The switch is also suitable for use in a variety of tissues, including roots, leaves, stems and reproductive tissues.

In a particularly preferred embodiment of the present invention, the gene switch of the present invention is used to control expression of genes which confer resistance herbicide resistance and/or insect tolerance to plants.

Recent advances in plant biotechnology have resulted in the generation of transgenic plants resistant to herbicide application, and transgenic plants resistant to insects. Herbicide tolerance has been achieved using a range of different transgenic strategies. One well documented example in the herbicide field is the use the bacterial xenobiotic detoxifying gene phosphinothricin acetyl transferase (PAT) from *Streptomyces hydroscopicus*. Mutated genes of plant origin, for example the altered target site gene encoding acetolactate synthase (ALS) from *Arabidopsis*, have been successfully utilised to

generate transgenic plants resistant to herbicide application. The PAT and ALS genes have been expressed under the control of strong constitutive promoter. In the field of insecticides, the most common example to-date is the use of the Bt gene.

We propose a system where genes conferring herbicide and/or insect tolerance would be expressed in an inducible manner dependent upon application of a specific activating chemical. This approach has a number of benefits for the farmer, including the following:

1. Inducible control of herbicide and/or insect tolerance would alleviate any risk of yield penalties associated with high levels of constitutive expression of herbicide and/or insect resistance genes. This may be a particular problem as early stages of growth where high levels of transgene product may directly interfere with normal development. Alternatively high levels of expression of herbicide and/or insect resistance genes may cause a metabolic drain for plant resources.
2. The expression of herbicide resistance genes in an inducible manner allows the herbicide in question to be used to control volunteers if the activating chemical is omitted during treatment.
3. The use of an inducible promoter to drive herbicide and/or insect resistance genes will reduce the risk of resistance becoming a major problem. If resistance genes were passed onto weed species from related crops, control could still be achieved with the herbicide in the absence of inducing chemical. This would particularly be relevant if the tolerance gene confirmed resistance to a total vegetative control herbicide which would be used (with no inducing chemical) prior to sowing the crop and potentially after the crop has been harvested. For example, it can be envisaged that herbicide resistance cereals, such as wheat, might outcross into the weed wild oats, thus conferring herbicide resistance to this already troublesome weed. A further example is that the inducible expression of herbicide resistance in sugar beet will reduce the risk of wild sugar beet becoming a problem. Similarly, in the field of insect control, insect resistance may well become a problem if the tolerance gene is constitutively expressed. The used of an inducible promoter will allow a greater range of insect resistance control mechanisms to be employed.

This strategy of inducible expression of herbicide resistance can be achieved with a pre-spray of chemical activator or in the case of slow acting herbicides, for example N-phosphonomethyl-glycine (commonly known as glyphosate), the chemical inducer can be

added as a tank mix simultaneously with the herbicide. Similar strategies can be employed for insect control.

This strategy can be adopted for any resistance ~~confering~~ **conferring** gene/corresponding herbicide combination, which is, or becomes, available. For example, the gene switch of the present invention can be used with:

1. Maize glutathione S-transferase (GST-27) gene (see our PCT International Patent Publication No. WO_90/08826), which confers resistance to chloroacetanilide herbicides such as acetochlor, metolachlor and alachlor.
2. Phosphinotricin acetyl transferase (PAT), which confers resistance to the herbicide commonly known as glufosinate.
3. Acetolactate synthase gene mutants from maize (see our International Patent Publication No. WO_90/14000) and other genes, which confer resistance to sulphonyl urea and imadazolinones.
4. Genes which confer resistance to glyphosate. Such genes include the glyphosate oxidoreductase **(GOX)** gene ~~(GOX)~~ (see International Patent Publication No. WO 92/00377); genes which encode for 5-enolpyruvyl-3-phosphoshikimic acid synthase (EPSPS), including Class I and Class II EPSPS, genes which encode for mutant EPSPS, and genes which encode for EPSPS fusion peptides such as that comprised of a chloroplast transit peptide and EPSPS (see for example EP 218 571, EP 293 358, WO_91/04323, WO_92/04449 and WO_92/06201); and genes which are involved in the expression of CP_Lyase.

Similarly, the strategy of inducible expression of insect resistance can be adopted for any ~~tolerance confering~~ **tolerance-conferring** gene which is, or becomes, available.

The gene switch of the present invention can also be used to controlled expression of foreign proteins in yeast and mammalian cells. Many heterologous proteins for many applications are produced by expression in genetically engineered bacteria, yeast cells and other ~~eucaryotic~~ **eukaryotic** cells such as mammalian cells.

As well as the obvious advantage in providing control over the expression of foreign genes in such cells, the switch of the present invention provides a further advantage in yeasts and mammalian cells where accumulation of large quantities of an heterologous protein can damage the cells, or where the heterologous protein is damaging such that expression for short periods of time is required in order to maintain the viability of the cells.

Such an inducible system also has applicability in gene therapy allowing the timing of expression of the therapeutic gene to be controlled. The present invention is therefore not only applicable to transformed mammalian cells but also to mammals *per se*.

A further advantage of the inducible system of the present invention in mammalian cells is that, because it is derived from a insect, there is less chance of it being effected by inducers which effect the natural mammalian steroid receptors.

In another aspect of the present invention the gene switch is used to switch on genes which produce potentially damaging or lethal proteins. Such a system can be employed in the treatment of cancer in which cells are transformed with genes which express proteins which are lethal to the cancer. The timing of the action of such proteins on the cancer cells can be controlled using the switch of the present invention.

The gene switch of the present invention can also be used to switch genes off as well as on. This is useful in disease models. In such a model the cell is allowed to grow before a specific gene(s) is switched off using the present invention. Such a model facilitates the study of the effect of a specific gene(s).

Again the method for producing such transgenic cells is not particularly germane to the present invention and any method suitable for the target cell may be used; such methods are known in the art, including cell specific transformation.

As previously mentioned, modulation of gene expression in the system appears in response to the binding of the HEcR to a specific control, or regulatory, DNA element. A schematic representation of the HEcR gene switch is shown in Figure 6. For ease of reference, the schematic representation only shows three main domains of the HEcR, namely the transactivation domain, DNA binding domain and the ligand binding domain. Binding of a ligand to the ligand binding domain enables the DNA binding domain to bind to the HRE resulting in expression (or indeed repression) of a target gene.

The gene switch of the present invention can therefore be seen as having two components: ~~. The~~ a first component comprising the HEcR and a second component comprising an appropriate HRE and the target gene. In practice, the switch may conveniently take the form of one or two sequences of DNA, at ~~. At~~ least part of the one sequence, or one sequence of the pair, encoding the HEcR protein. Alternatively, the nucleic acid encoding the HEcR can be replaced by the protein/ polypeptide itself.

Not only does the switch of the present invention have two components, but also one or more of the domains of the receptor can be varied producing a chimeric gene

switch. The switch of the present invention is very flexible and different combinations can be used in order to vary the result/to optimise the system. The only requirement in such chimeric systems is that the DNA binding domain should bind to the hormone response element in order to produce the desired effect.

The glucocorticoid steroid receptor is well characterised and has been found to work well in plants. A further advantage of this receptor is that it functions as a homodimer. This means that there is no need to express a second protein such as the ultraspiracle in order to produce a functional receptor. The problem with the glucocorticoid steroid receptor is that ligands used to activate it are not compatible with agronomic practice.

In a preferred aspect of the present invention the receptor comprises glucocorticoid receptor DNA binding and transactivation domains with a *Heliothis* ligand binding domain according to the present invention. The response unit preferably comprising the glucocorticoid hormone response element and the desired effect gene. In the Examples, for convenience, this effect gene took the form of a reporter gene. However, in non-test or non-screen situations the gene will be the gene which produces the desired effect, for example produces the desired protein. This protein may be a natural or exogenous protein. It will be appreciated that this chimeric switch combines the best features of the glucocorticoid system, whilst overcoming the disadvantage of only being inducible by a steroid.

In another preferred embodiment, the *Heliothis* ligand binding domain is changed, and preferably replaced with a non-*Heliothis* ecdysone receptor ligand binding domain. For example, we have isolated suitable sequences from *Spodoptera exigua*.

Thus, according to another aspect of the present invention there is provided DNA having the sequence shown in ~~Seq ID No.~~ **SEQ ID NO:** 6.

According to another aspect of the present invention there is provided DNA having part of the sequence shown in ~~Seq ID No.~~ **SEQ ID NO:** 6, which encodes for the *Spodoptera* ecdysone ligand binding domain.

According to another aspect of the present invention there is provided DNA having part of the sequence shown in ~~Seq ID No.~~ **SEQ ID NO:** 6, which encodes for the *Spodoptera* ecdysone hinge domain.

The present invention also provides the polypeptides coded for by the above DNA sequences of ~~Seq ID No.~~ **SEQ ID NO:** 6.

A further advantage with such chimeric systems is that they allow you to choose the promoter which is used to drive the effector gene according to the desired end result. For example, placing the foreign gene under the control of a cell specific promoter can be particularly advantageous in circumstances where you wish to control not only the timing of expression, but also which cells expression occurs in. Such a double control can be particularly important in the areas of gene therapy and the use of cytotoxic proteins.

Changing the promoter also enables gene expression to be up- or down-regulated as desired.

Any convenient promoter can be used in the present invention, and many are known in the art.

Any convenient transactivation domain may also be used. The transactivation domain VP16 is a strong activator from Genentech Inc., and is commonly used when expressing glucocorticoid receptor in plants. Other transactivation domains derived for example from plants or yeast may be employed.

In a preferred embodiment of the present invention, the DNA binding domain is the glucocorticoid DNA binding domain. This domain is commonly a human glucocorticoid receptor DNA binding domain. However, the domain can be obtained from any other convenient source, for example, rats.

According to another aspect of the present invention there is provided a method of selecting compounds capable of being bound to an insect steroid receptor superfamily member comprising screening compounds for binding to a polypeptide or fusion polypeptide of the present invention, and selecting said compounds exhibiting said binding.

According to another aspect of the present invention there is provided a compound selected using the method of the present invention.

According to another aspect of the present invention there is provided an agricultural or pharmaceutical composition comprising the compound of the present invention.

According to yet another aspect of the present invention there is provided the use of the compound of the present invention as a pesticide, pharmaceutical and/or inducer of the switch. It will be appreciated that such inducers may well be useful as insecticides in themselves.

According to a further aspect of the present invention there is provided a method of producing a protein or peptide or polypeptide comprising introducing into a cell of the present invention, a compound which binds to the ligand binding domain in said cell.

Various preferred features and embodiments of the present invention will now be described by way of non-limiting example with reference to the accompanying examples and figures, in which figures:

Brief Description of the Drawings

Figure 1 ~~(Sequence ID No. 1)~~ shows the DNA sequence amplified from first strand cDNA made from mRNA isolated from *Heliothis virescens* Fourth instar larvae **(SEQ ID NO: 1)** The underlined sequences refer to the position of the degenerate oligonucleotides. At the 5' end the sequence matches that of the oligonucleotide while at the 3' end 12 nucleotides of the original oligonucleotide are observed;

Figure 2 (~~Sequence ID No. 2)~~ shows the DNA sequence contained within the clone pSK19R **(SEQ ID NO: 2)** isolated from a random primed cDNA *Heliothis virescens* library; Sequence is flanked by EcoRI sites;

Figure 3 ~~(Sequence ID No. 3)~~ shows the DNA sequence contained within the clone pSK16.1 **(SEQ ID NO: 3)** isolated from a random primed cDNA *Heliothis virescens* library;

Figure 4 ~~(Sequence ID No. 4)~~ **presents the** DNA sequence of 5'_RACE products (in bold) fused to sequence of clone pSK16.1. The ORF (open reading frame**; SEQ ID NO: 4)** giving rise to the *Heliothis virescens* ecdysone receptor protein sequence **(SEQ ID NO: 5)** is shown under the corresponding DNA sequence;

Figure 5 ~~(Sequence ID No. 5)~~ shows the protein sequence alignment of the ecdysone receptors DmEcR (*Drosophila melanogaster*; **SEQ ID NO: 8**), CtEcR (*Chironomus tentans*; **SEQ ID NO: 9**), BmEcR (*Bombyx mori*; **SEQ ID NO: 10**), MsEcR (*Manduca sexta*; SEQ ID NO: 11), AaEcR (*Aedes ~~aegipti~~ **aegypti***; **SEQ ID NO: 12**) and HvEcR (*Heliothis virescens*; **SEQ ID NO: 5**). "*" indicates conserved amino acid residue. "." indicates a conservative amino acid exchange;

Figure 6 shows a model of an embodiment of the glucocorticoid/*Heliothis* ecdysone chimeric receptor useable as a gene switch;

Figure 7 shows a plasmid map of the clone pcDNA319R. The three other mammalian expression vectors were constructed in the same way and look similar but for the size of the insert;

Figure 8 shows a plasmid map of the reporter construct used to analyse the activity of the *Heliothis virescens* ecdysone receptor;

Figure 9 is a graph which shows the effect of Muristerone A and RH5992 in reporter activity in HEK293 cells co-transfected with pcDNA3H3KHEcR alone (filled bars) or with αRXR (stripped bars);

Figure 10 shows a plasmid map of the Maize expression vector containing the Glucocorticoid receptor (HG1 or pMF6HG1PAT);

Figure 11 shows a plasmid map of the maize expression vector containing the chimeric glucocorticoid/*Drosophila* ecdysone receptor pMF6GREcRS;

Figure 12 shows a plasmid map of the maize expression vector containing the chimeric glucocorticoid/*Heliothis* ecdysone receptor pMF6GRHEcR;

Figure 13 shows a plasmid map of the plant reporter Plasmid containing the glucocorticoid response elements fused to the -60 S35CaMV promoter fused to GUS, p221.9GRE6;

Figure 14 shows a plasmid map of the plant reporter plasmid containing the glucocorticoid response elements fused to the -46 S35CaMV promoter fused to GUS, p221.10GRE6;

Figure 15 shows a graph showing the effect of Muristerone A and Dexamethasone in Maize AXB protoplasts transformed with pMF6HG1PAT (GR) and p221.9GRE6 (reporter);

Figure 16 shows a graph showing the effect of Muristerone A and Dexamethasone in Maize AXB protoplasts transformed with pMF6GREcRS (effector) and p221.9GRE6 (reporter);

Figure 17 shows a graph showing the effect of Muristerone A and Dexamethasone in Maize AXB protoplasts transformed with pMF6GRHEcR (effector) and p221.9GRE6 (reporter);

Figure 18 shows a graph showing the effect of RH5849 in Maize AXB protoplasts transformed with pMF6GREcRS (effector) and p221.9GRE6 (reporter);

Figure 19 shows a graph showing the effect of RH5992 in Maize AXB protoplasts transformed with pMF6GREcRS (effector) and p221.9GRE6 (reporter);

Figure 20 shows a graph showing the effect of RH5992 in Maize AXB protoplasts transformed with pMF6GRHEcR (effector) and p221.9GRE6 (reporter);

Figure 21 shows a graph which shows the dose response effect of RH5992 in Maize AXB protoplasts transformed with pMF6GRHEcR (effector) and p221.9GRE6 (reporter);

Figure 22 shows a plasmid map of the tobacco expression vector containing the chimeric glucocorticoid/ *Drosophila* ecdysone receptor, pMF7GREcRS;

Figure 23 shows a plasmid map of the tobacco expression vector containing the chimeric glucocorticoid/ *Heliothis* ecdysone receptor, pMF7GRHEcR;

Figure 24 shows a graph which shows the effect of RH5992 in Tobacco mesophyll protoplasts transformed with pMF6GRHEcR (Effector) and p221.9GRE6 (reporter);

Figure 25 shows a plasmid map of the mammalian expression vector containing the chimeric glucocorticoid/*Heliothis* ecdysone receptor, pcDNA3GRHEcR;

Figure 26 shows a plasmid map of the reporter plasmid pSWGRE4;

Figure 27 shows a graph which shows a RH5992 dose response curve of CHO cells transfected with pcDNA3GRHEcR and pSWGRE4;

Figure 28 shows a graph which shows the effect of Muristerone A and RH5992 on HEK293 cells co-transfected with pcDNA3GRHEcR and pSWGRE4;

Figure 29 shows a plasmid map of the binary vector ES1;

Figure 30 shows a plasmid map of the binary vector ES2;

Figure 31 shows a plasmid map of the binary vector ES3;

Figure 32 shows a plasmid map of the binary vector ES4;

Figure 33 shows a plasmid map of the effector construct TEV-B112 made to express the HEcR ligand binding domain in yeast;

Figure 34 shows a plasmid map of the effector construct TEV8 made to express the HEcR ligand binding domain in yeast;

Figure 35 shows a plasmid map of the effector construct TEVVP16-3 made to express the HEcR ligand binding domain in yeast;

Figure 36 shows a plasmid map of the mammalian expression vector containing the chimeric glucocorticoid VP16/*Heliothis* ecdysone receptor, pcDNA3GRVP16HEcR;

Figure 37 shows a plasmid map of the maize expression vector containing the chimeric glucocorticoid VP16/*Heliothis* ecdysone receptor, pMF6GRVP16HEcR;

Figure 38 shows a plasmid map of the maize expression vector containing the chimeric glucocorticoid VP16/*Heliothis* ecdysone receptor, pMF7GRVP16HEcR;

Figure 39 shows a graph which shows the effect of RH5992 in Maize AXB protoplasts transformed with pMF6GRVP16HEcR (effector) and p221.9GRE6 (reporter);

Figure 40 ~~(Sequence ID No. 6)~~ shows the DNA sequence of the hinge and ligand binding domains of the *Spodoptera exigua* ecdysone receptor (SEQ ID NO: 6);

Figure 41 ~~(Sequence ID No. 7)~~ shows the protein sequence alignment of the *Heliothis 19R* (SEQ ID NO: 13) and *Spodoptera* SEcR *Taq* clone hinge and ligand binding domains (SEQ ID NO: 7). "*" indicates conserved amino acid residue. "." indicates a conservative amino acid exchange;

Figure 42 shows a graph which shows the effect of RH5992 on Tobacco mesophyll protoplasts transformed with pMF7GRHEcR (effector) and either p221.9GRE6 (Horizontal strips) or p221.10GRE6 (vertical strips).

Example I - Cloning of the *Heliothis* Ecdysone Receptor

A. Probe generation

The rational behind the generation of the probe to isolate *Heliothis* homologues to the steroid/thyroid receptor superfamily members was based on comparing the sequences of developmentally regulated steroid/thyroid receptor superfamily members. The sequences available showed a highly conserved motif within the DNA binding domain of the RAR and THR (thyroid) receptors. The motifs were used to design degenerate oligonucleotides for PCR amplification of sequences derived from cDNA template produced from tissue expected to express developmentally regulated steroid/thyroid receptor superfamily members (i.e. larval tissues).

The sense oligonucleotide is based on the peptide sequence CEGCKGFF **(SEQ ID NO: 14)** which at the DNA level yields an ~~oligonucletide~~ oligonucleotide with degeneracy of 32 as shown below:

```
ZnFA5' 5' TGC GAG GGI TGC AAG GAI TTC TT 3' (SEQ ID NO: 15)
          T   A       T   A       T
```

The antisense oligonucleotide is based on the reverse complement nucleotide sequence derived from the peptide:

CQECRLKK **(SEQ ID NO: 16)**
S R

for which four sets of degenerate oligos were made. Namely:

ZnFA3' 5' TTC TTI AGI CGG CAC TCT TGG CA 3' **(SEQ ID NO: 17)**
T A T C A

ZnFB3' 5' TTC TTI AAI CGG CAC TCT TGG CA 3' **(SEQ ID NO: 18)**
T A T C A

ZnFC3' 5' TTC TTI AGI CTG CAC TCT TGG CA 3' **(SEQ ID NO: 19)**
T A T C A

ZnFD3' 5' TTC TTI AAI CTG CAC TCT TGG CA 3' **(SEQ ID NO: 20)**
T A T C A

The PCR amplification was carried out using a randomly primed cDNA library made from mRNA isolated from 4th and 5th instar *Heliothis virescens* larvae. The amplification was performed using 10^8 ~~pfus~~ **plaque forming units (pfu)** in 50_mM KCl. 20_mM Tris HCl pH_8.4, 15_mM MgCl2, 200_mM dNTPs (an equimolar mixture of dCTP, dATP, dGTP and dTTP), 100_ng of ZnFA5' and ZnF3' mixture. The conditions used in the reaction followed the hot start protocol whereby the reaction mixture was heated to 94°C for 5 minutes after which 1 U of Taq polymerase was added and the reaction allowed to continue for 35 cycles of 93°C for 50 seconds, 40°C for 1 minute and 73°C for 1 minute 30 seconds. The PCR products were fractionated on a 2% (w/v) agarose gel and the ragment migrating between 100 and 200 bp markers was isolated and subcloned into the vector pCRII (Invitrogen). The sequence of the insert was determined using Sequenase (USB).

The resulting sequence was translated and a database search carried out. The search recovered sequences matching to the DNA binding domain of the *Drosophila* ecdysone receptor, retinoic acid receptor and the thyroid receptor. Thus, the sequence of the insert in this plasmid, designated pCRIIZnf, is a *Heliothis* ecdysone cognate sequence (Figure 1) and was used to screen a cDNA library in other to isolate the complete open reading frame.

B. Library screening

The randomly primed cDNA 4th/5th Instar *Heliothis virescens* library was plated and replicate filter made from the plates. The number of plaques plated was 500,000. The

insert fragment of pCRIIZnf was reamplified and 50_ng were end labelled using T4 Polynucleotide Kinase (as described in Sambrook et al 1990).

The filters were prehybridised using 0.25%(w/v) Marvel, 5 X SSPE and 0.1%_(w/v) SDS at 42°C for 4 hours. The solution in the filters was ~~ten~~ **then** replaced with fresh solution and the denatured probe added. The hybridisation was carried out overnight at 42°C after which the filters were washed in 6 X SSC + 0.1%_(w/v) SDS at 42°C followed by another wash at 55°C. The filters ~~were~~ was exposed to X-ray film (Kodak) for 48 hours before processing.

The developed film indicated the presence of one strong positive signal which was plaque purified and further characterised. The lambda ZAP II phage was in vivo excised (see Stratagene Manual) and the sequence determined of the resulting plasmid DNA. The clone known as pSK19R (or 19R) contained a 1.933 **kilobase (kb)** cDNA fragment with an open reading frame of 467 amino acids (Figure 2). pSK19R was deposited with the NCIMB on 20 June 1995 and has been accorded the deposit No. NCIMB 40743.

Further analysis of pSK19R revealed that a 340 bp EcoRI fragment mapping at the 5' end of pSK19R has strong and significant similarities to a *Drosophila* cDNA encoding glyceraldehyde-3-phosphate dehydrogenase. In order to isolate the correct 5'_end sequence belonging to *Heliothis*, the random primed library was re-screened using a probe containing the 5' end of the pSK19R belonging to *Heliothis* ecdysone receptor. The probe was made by PCR using the sense oligonucleotide HecRH3C (5' aattaagcttccaccatgccgttaccaatgccaccgaca 3'; SEQ ID NO:21) and antisense oligonucleotide HecrNdeI (5' cttcaaccgacactcctgac 3'**; SEQ ID NO: 22**). The PCR was carried out as described by Hirst et al., 1992), where the amount of radioisotope used in the labelling was 50 ~~uCi~~ **µCi** of a ^{32}P-dCTP and the PCR was cycled for 1 minute at 94°C, 1 minute at 60°C and 1 minute at 72°C for 19 cycles. The resulting 353_bp radio labelled DNA fragment was denatured and added to prehybridised filters as described for the isolation of pSK19R. The library filters were made from 15 plates each containing 50,000 pfus. The library filters were hybridised at 65°C and washed in 3X_SSPE + 0.1%_SDS at 65°C twice for 30 minutes each. The filters were further washed with 1X_SSPE + 0.1% SDS for 30 minutes and exposed to X-ray film (Kodak) overnight. The film was developed and 16 putative positive plaques were picked. The plaques were re-plated and hybridised under the exact same conditions as the primary screen resulting in only one strong positive. The strong positive was consistently recognised by the probe and was

plaque purified and *in vivo* excised. The resulting plasmid pSK16.1 was sequenced (~~Seq ID 3~~ SEQ ID NO: 3) which revealed that the 5' end of the clone extended by 205 bp and at the 3' end by 653 bp and resulting in a DNA insert of 2.5 kb. Conceptual translation of the 205 bp yielded 73 amino acids with high similarity to the *Drosophila*, *Aedes ~~aegipti~~ aegypti*, *Manduca* and *Bombyx* sequences of the ~~ecdysone~~ ecdysone receptor B1 isoform. However, the whole of the 5' end sequence is not complete since a Methionine start site was not found with a stop codon in frame 5' of the methionine. In order to isolate the remainder of the 5' end coding sequences a 5' RACE protocol (Rapid Amplification of cDNA Ends) was carried out using the BRL-GIBCO 5' RACE Kit. Two types of cDNA were synthesised where the first one used a specific oligonucleotide:

16PCR2A 5' cagctccaggccgccgatctcg 3' **(SEQ ID NO: 23)**

and the second type used random hexamers (oligonucleotide containing 6 random nucleotides). Each cDNA was PCR amplified using the oligonucleotides anchor primer:

BRL-GIBCO 5' cuacuacuacuaggccacgcgtcgactagtacgggiigggiigggiig 3' **(SEQ ID NO: 24)**

and 16PCR2A and cycled for 1 minute at 94°C, 1 minute at 60°C and 1 minute at 72°C for 35 cycles. The reaction conditions were 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM $MgCl_2$, 400 nM of each anchor and 16PCR2A primers, 200 mM dNTPs (dATP, dCTP, dGTP and dTTP) and 0.02 U/ml *Taq* DNA polymerase. Dilutions of 1:50 of the first PCR reactions were made and 1 ml was used in a second PCR with oligonucleotides UAP:

(Universal Amplification Primer) 5' caucaucaucauggccacgcgtcgactagtac 3' **(SEQ ID NO: 25)**

and 16RACE2: (5' acgtcacctcagacgagctctccattc 3' **(SEQ ID NO: 26)**.

The conditions and cycling were the same as those followed for the first PCR. Samples of each PCR were run and a Southern blot carried out which was probed with a 5' specific primer: (16PCR1; 5' cgctggtataacaacggaccattc 3' **(SEQ ID NO. 27)**.

This primer is specific for the 5' most sequence of pSK16.1 and was hybridised at 55°C using the standard hybridisation buffer. The filter was washed at 55°C 3 times in 3X SSPE + 0.1% SDS and exposed to X-ray film for up to 6 hours. The developed film revealed bands recognised by the oligonucleotide migrating at 100 bp and 500 bp (relative to the markers). A sample of the PCR reaction (4 in total) was cloned into the pCRII vector in the TA cloning kit (Invitrogen). Analysis of 15 clones from 4 independent PCRs yielded sequence upstream of pSK16.1 (Figure 4).

Translation of the ORF results in a 575 amino acid protein with high similarity in the DNA and ligand binding domains when compared to the ecdysone receptor sequences of *Drosophila* , *Aedes aegypti*, *Chironomus tentans*, *Manduca sexta* and *Bombyx mori* (Figure 5). Interestingly, the N-terminal end of the *Heliothis* sequence has an in frame ~~methinonine~~ **methionine** start which is 20 amino acids longer that that reported for *Drosophila* , *Aedes aegypti* and *Manduca sexta*. However, the extended N-terminal end in the *Heliothis* EcR does not have similarity to that of *Bombyx mori*. Finally, the C-terminal ends of the different B1 isoform ecdysone receptor sequences diverge and do not have significant similarity.

C. Northern Blot Analysis

The sequence identified by screening the library is expected to be expressed in tissues undergoing developmental changes, thus mRNA from different developmental stages of *H. virescens* were was isolated and a Northern blot produced. The mRNAs were isolated from eggs, 1st, 2nd, 3rd, 4th and 5th instar larvae, pupae and adults. The Northern blot was hybridised with an NdeI/XhoI DNA fragment from pSK19R encompassing the 3'_end of the DNA binding domain through to the end of the ligand binding domain. The hybridisation was carried out in 1%_(w/v)_Marvel, 5X SSPE, 0.1% (w/v) SDS at 65°C for 18 to 24 hours. The filters were washed in 3X SSPE + 0.1% (w/v) SDS and 1X SSPE + 0.1% (w/v) SDS at 65°C. The filter was blotted dry and exposed for one to seven days. The gene recognises two transcripts (6.0 and 6.5 kb) which appear to be expressed in all stages examined, however, the levels of expression differ in different stages. It should be noted that the same two transcripts are recognised by probes specific to the DNA binding domain and the ligand binding domain, indicating that the two transcripts arise from the same gene either by alternative splicing or alternative use of polyadenylation sites.

In summary, adult and 5th instar larvae have lower levels of expression while all other tissues have substantial levels of expression.

Example II - Expression of *Heliothis* ecdysone receptor in Mammalian cells

To demonstrate that the cDNA encodes a functional ecdysone receptor, effector constructs were generated containing the HEcR under the control of the CMV (cytomegalovirus) promoter, and the DNA expressed in mammalian cells.

A. Effector constructs

A first mammalian expression plasmid was constructed by placing a HindIII/NotI pSK19R fragment into the pcDNA3 HindIII/NotI vector resulting in pcDNA319R (Figure 7).

A second effector plasmid was constructed wherein the non-coding region of the cDNA 19R was deleted and a consensus Kozak sequence introduced. The mutagenesis was carried out by PCR amplifying a DNA fragment with the oligo HecRH3C: 5'aattaagcttccaccatgccgttaccaatgccaccgaca 3' **(SEQ ID NO: 21)** containing a unique HindIII restriction enzyme recognition site followed by the mammalian Kozak consensus sequence, and HecRNdeI: 5'cttcaaccgacactcctgac 3' **(SEQ ID NO: 22)**.

The resulting 353 bp PCR fragment was restriction enzyme digested with HindIII and NdeI, gel purified and ligated with 19R NdeI/NotI fragment into a pcDNA3 HindIII/NotI vector resulting in pcDNA3HecR.

A third effector construct was made with the 5' end sequences of pSK16.1 by PCR. The PCR approach involved PCR amplifying the 5' end sequences using a 5' oligonucleotide containing a HindIII restriction cloning site, the Kozak consensus sequence followed by nucleotide sequence encoding for a Methionine start and two Arginines to be added to the 5' end of the amplified fragment: (16H3K 5' attaagcttgccgccatgcgccgacgctggtataacaacggaccattc 3'; **SEQ ID NO: 28)**, the 3' oligonucleotide used was HecrNdeI. The resulting fragment was restriction enzyme digested, gel purified and subcloned with an NdeI/NotI 19R fragment into pcDNA3 NdeI/NotI vector. The plasmid was named pcDNA3H3KHEcR.

A fourth effector construct was produced which contains the extended N-terminal end sequence obtained from the 5' RACE experiment. Thus, a PCR approach was followed to introduce the new 5' end sequences in addition to a consensus Kozak sequence and a HindIII unique cloning sequence. The sense oligonucleotide used was RACEH3K: 5' attaagcttgccgccatgtccctcggcgctcgtggatac 3' **(SEQ ID NO: 29)**, while the antisense primer was the same as that used before (HecrNdeI). The cloning strategy was the same as used for the pcDNA3H3KHEcR to give rise to pcDNA3RACEH3KHEcR.

The PCR mutagenesis reactions were carried out in the same manner for all constructs. The PCR conditions used were 1 minute at 94°C, 1 minute at 60°C and 1 minute at 72°C for 15 cycles. The reactions conditions were 50 mM Tris-HCl (pH 8.4), 25

mM KCl, 200_mM dNTPs (dATP, dCTP, dGTP and dTTP), 200_nM of each oligonucleotide and 2.5U/Reaction of *Taq* DNA polymerase. For each construct at least 5 ~~independant~~ **independent** PCR reactions were carried out and several clones were sequenced to insure that at least one is mutation free.

B. Reporter construct

The reporter plasmid to be co-transfected with the expression vector contained 4 copies of the Hsp27 ecdysone response element (Riddihough and Pelham, 1987) fused to ~~B~~β-globin promoter and the ~~B~~β-Galactosidase gene. The tandem repeats of the ecdysone response element were synthesised as two complementary oligonucleotides which when annealed produced a double ~~standed~~ **stranded** DNA molecule flanked by an SpeI site at the 5' end and a ClaI site at the 3' end:

Recr3A:

5' ctagtagacaagggttcaatgcacttgtccaataagcttagacaagggttcaatgcacttgtccaatgaattcagacaaggg ttcaatgcacttgtccaatctgcagagacaagggttcaatgcacttgtccaatat 3' **(SEQ ID NO: 30)**

Recr3B:

5' cgatattggacaagtgcattgaacccttgtctctgcagattggacaagtgcattgaacccttgtctgaattcattggacaagt gcattgaacccttgtctaagcttattggacaagtgcattgaacccttgtcta 3' **(SEQ ID NO: 31)**.

The resulting 135_bp DNA fragment was ligated to the vector pSWBGAL SpeI/ClaI resulting in pSWREcR4 (Figure 8). The co-transfection of the two plasmids should result in B-galactosidase activity in the presence of ligand. The experiment relies upon the presence of RXR (a homologue of ultraspiracle) in mammalian cells for the formation of an active ecdysone receptor.

C. Mammalian transfection methods

Transfections of mammalian cell lines (CHO-K1 Chinese hamster ovary)- ATCC number CCL61 or cos-1 (Monkey cell line) were performed using either calcium phosphate precipitation (Gorman, Chapter 6 of "DNA cloning: a practical approach. Vol 2 D.M. Glover ed/.(1985) IRL Press, Oxford) or using LipofectAMINE (Gibco BRL Cat. No. 18324-012, following manufacturer's instructions). Human Epithelial Kidney 293 cells were transfected using analogous methods.

D. Results - Native HEcR drives transient reporter gene expression in mammalian cells

Co-transfection of pcDNA3H3KHEcR (Effector) and reporter constructs into Human Epithelial Kidney 293 cells (HEK293) in the presence of either Muristerone A or RH5992 resulted in a 2-3 fold induction of reporter activity compared to the no chemical controls (Figure 9). The HEK293 cells were used since they are known to have constitutive levels of αRXR which have been demonstrated to be necessary for *Drosophila* EcR activation by Muristerone A (Yao~~,~~ *et al.*, 1993). Moreover, to further investigate the need for RXR interactions, a αRXR was co-transfected into HEK293 cells (along with the effector and reporter) resulting in a 9-fold induction of reporter activity compared to the untreated cells (Figure 9). The co-transfection of αRXR with reporter and effector increased by four fold the reporter activity compared to cells transfected with effector and reporter alone. Induction was observed both in the presence of either Muristerone A or RH5992. These data clearly demonstrate that the cDNA HEcR encodes a functional ecdysone receptor. Moreover, ~~The~~ **the** ability of HEcR to complex with αRXR and bind Muristerone A or RH5992 provide evidence for the usage of the entire HEcR as a component of a mammalian gene switch. In particular, it offers the advantage of reducing uninduced expression of target gene since ecdysone receptor and response elements are not present in mammalian cells.

Example III - Chimeric constructs and ligand validation in Maize Protoplasts

In order to apply the ecdysone receptor as an inducible system it was deemed necessary to simplify the requirements of the system by avoiding the need of a heterodimer formation to obtain an active complex. The glucocorticoid receptor is known to form homodimers and chimeric constructs of the glucocorticoid receptor transactivating and DNA binding domains fused to the ecdysone receptor hinge and ligand binding domains have been shown to be active as homodimers in mammalian cells in the presence of Muristerone A (an ecdysone agonist) (Christopherson et al., 1992). However, the chimeric receptor is not responsive to 20-hydroxyecdysone (Christopherson et al., 1992).

The analysis of the activation of the glucocorticoid/*Heliothis* ecdysone chimeric receptor entailed the production of two other control effector constructs. The first one of the constructs contained the intact glucocorticoid receptor while the second one contained a glucocorticoid/*Drosophila* ecdysone chimeric receptor.

A. Effector constructs

(i) Glucocorticoid receptor Maize expression construct

The glucocorticoid receptor DNA for the Maize transient expression construct was produced via the polymerase chain reaction (PCR) of Human Fibrosarcoma cDNA (HT1080 cell line, ATCC#CCl121) library (Clontech~~)~~ ~~(~~; see Hollenberg *et al.*, 1985). The PCR approach taken was to amplify the 2.7_kb fragment encoding the glucocorticoid receptor in two segments. The first segment entails the N-terminal end up to and including the DNA binding domain while the second fragment begins with the hinge region (amino acid 500) thought to the end of the reading frame. Thus, the PCR primer for the N-terminal end segment was designed to contain an EcoRI site and the Kozak consensus sequence for translation initiation: GREcoRI 5' attgaattccaccatggactccaaagaatcattaactc 3' **(SEQ ID NO: 32)**.

The 3' end primer contains an XhoI site in frame with the reading frame at amino acid 500 of the published sequence: GRXhoI 5' gagactcctgtagtggcctcgagcattccttttatttttttc 3' **(SEQ ID NO: 33)**.

The second fragment of the glucocorticoid receptor was produced with a 5' end oligonucleotide containing an XhoI site in frame with the open reading frame at the beginning of the hinge region (amino acid 500): GRHinge 5' attctcgagattcagcaggccactacaggag 3' **(SEQ ID NO: 34)** while the 3' end oligonucleotide contained an EcoRI site 400 bp after the stop codon: GRStop; 5' attgaattcaatgctatcgtaactatacaggg 3' **(SEQ ID NO: 35)**.

The glucocorticoid receptor PCR was carried out using Vent polymerase (Biolabs) under hot start conditions followed by 15 cycles of denaturing (94°C for 1 minute), annealing (66°C for 1 minute) and DNA synthesis (72°C for 3 minutes). The template was produced by making first strand cDNA as described in the TA cloning kit (Invitrogen) after which the PCR was carried out in 10_mM KCl, 10_mM $(NH_4)_2SO_4$, 20_mM TRIS-HCl pH_8.8, 2 mM $MgSO_4$, 0.1% (v/v) Triton X-100, 200 mM dNTPs, 100_ng of each Primer and 2 U of Vent Polymerase. The PCR products ~~was~~ **were** restriction enzyme digested with EcoRI and XhoI and subcloned into pBluescript SK (pSK) EcoRI. The resulting plasmid pSKHGI was sequenced and found to lack any mutations from the published sequences (apart from those introduced in the PCR primers) (Hollenberg et al., 1985).

The 2.7 kb EcoRI fragment was subcloned into the vector pMF6PAT EcoRI resulting in pMF6HGIPAT (Figure 10).

(ii) Maize expression construct containing a Glucocorticoid/ *Drosophila* ecdysone chimeric receptor.

The glucocorticoid receptor portion of the chimeric receptor was isolated from pSKHGI by producing a 1.5_kb BamHI/XhoI restriction fragment containing the N-terminal end up to and including the DNA binding domain.

The *Drosophila* ecdysone receptor portion was isolated through PCR of first stand cDNA prepared from *Drosophila* adult mRNA. The PCR was carried out using a 5' oligonucleotide containing a SalI site (~~ie.~~ **i.e.** *Drosophila* ecdysone receptor contains a XhoI site at the end of the ligand binding domain) which starts at the ~~begining~~ **beginning** of the hinge region: amino acid 330:

Ecr8 attgtcgacaacggccggaatggctcgtcccggag 3' **(SEQ ID NO: 36)**.

The 3' end oligonucleotide contains a BamHI site adjacent to the stop codon: EcRstop 5' tcgggctttgttaggatcctaagccgtggtcgaatgctccgacttaac 3' **(SEQ ID NO: 37)**.

The PCR was carried out under the conditions described for the amplification of the Glucocorticoid receptor and yielded a 1.6 kb fragment. The fragment was introduced into pSK SalI/BamHI and the sequence determined and compared to the published one (Koelle et al.,_1991).

The maize transient expression plasmid was produced by introducing into pMF6 BamHI vector the 1.5 kb BamHI/XhoI glucocorticoid receptor fragment and the 1.6 kb SalI/BamHI *Drosophila* receptor portion to yield the chimeric plasmid pMF6GREcRS (Figure 9).

(iii) Construction of the Glucocorticoid/*Heliothis* ecdysone chimeric receptor Maize transient expression plasmid.

The Glucocorticoid receptor portion of the chimera was produced as described in Example II(ii). The production of the *Heliothis* ecdysone receptor portion involves the introduction of a SalI recognition site at the DNA binding/hinge domain junction (amino acid 229). The addition of the SalI site:

Hecrsal 5'attgtcgacaaaggcccgagtgcgtggtgccggag **3' (SEQ ID NO: 38)**

was achieved via PCR mutagenesis making use of a unique AccI site 107 bp downstream of the ~~juction~~ junction point (or 1007 bp relative to ~~SeqID No~~ **SEQ ID NO:** 4): Hecracc 5' tcacattgcatgatgggaggcatg 3' **(SEQ ID NO: 39)**.

The PCR was carried out using *Taq* polymerase (2.5 U) in a reaction buffer containing 100_ng of template DNA (pSK19R), 100_ng of Hecrsal and Hecracc, 20 mM TRIS-HCl pH 8.4, 50_mM KCl, 10_mM $MgCl_2$, 200_mM dNTPs. The reaction was carried out with an initial denaturation of 3 minutes followed by 15 cycles of denaturation (1 minute at 94°C), annealing (1 minute at 60°C) and DNA synthesis (1 minute at 72°C). The DNA was restriction enzyme digested and subcloned into pSK SalI/SacI with the 1.2 kb AccI/SacI 3' end HecR fragment to yield pSK HeCRDEF (or containing the hinge and ligand binding domains of the *Heliothis* ecdysone receptor). The construction of the maize transient expression plasmid containing the Glucocorticoid/*Heliothis* ecdysone chimeric receptor involved the ligation of pMF6 EcoRI/SacI with the 1.5 kb EcoRI/XhoI fragment of Glucocorticoid receptor N-terminal end and the 1.2 kb SalI/SacI fragment of pSk HEcRDEF to yield pMF6GRHEcR (Figure 10).

B. Reporter plasmids

Two reporter plasmids were made by inserting the into p221.9 or p221.10 BamHI/HindIII vectors two pairs or oligonucleotides containing six copies of the glucocorticoid response element (GRE). The two sets of oligonucleotides were designed with restriction enzyme recognition sites so as to ensure insertion of the two pairs in the right orientation. The first oligonucleotide pair GRE1A/B is 82 ~~nucletides~~ **nucleotides** long and when annealed result in a DNA fragment flanked with a HindIII site at the 5' end and a SalI site at the 3' end:

GRE1A

5' agcttcgactgtacaggatgttctagctactcgagtagctagaacatcctgtacagtcgagtagctagaacatcctgtacag 3' **(SEQ ID NO: 40)**

GRE1B

5' tcgactgtacaggatgttctagctactcgactgtacaggatgttctagctactcgagtcgctagaacatcctgtacagtcga 3' **(SEQ ID NO: 41)**.

The second pair of oligonucleotides is flanked by a SalI site at the 5' end and a BamHI site at the 3' end:

GRE2A

5' tcgactagctagaacatcctgtacagtcgagtagctagaacatcctgtacagtcgagtagctagaacatcctgtacag 3' **(SEQ ID NO: 42)**

GRE2B

5' gatcctgtacaggatgttctagctactcgactgtacaggatgttctagctactcgactgtacaggatgttctagctag 3' **(SEQ ID NO: 43)**.

The resulting plasmids were named p221.9GRE6 (Figure 13) and p221.10GRE6 (Figure 14) (used in later Example). The difference between p221.9 and p221.10 plasmids is that p221.9 contains the -60 35SCaMV minimal promoter while p221.10 (p221.10GRE6) contains the -46 35SCaMV minimal promoter.

C. Method

Protoplasts were isolated from a maize suspension culture derived from BE70 x A188 embryogenic callus material, which was maintained by subculturing twice weekly in $MS0.5_{mod.}$ (MS medium supplemented with 3% sucrose, 690 mg/l proline, 1g/l myo-inositol, 0.2g/l casein acid hydrolysate, 0.5 mg/l 2,4-D, pH_5.6). Cells from suspensions two days post subculture were digested in enzyme mixture (2.0% Cellulase RS, 0.2% Pectolyase Y23, 0.5_M Mannitol, 5_mM $CaCl_2 \cdot 2H_2O$, 0.5% MES, pH_5.6, ~660_mmol/kg) using ~10_ml/g cells, incubating at 25°C, dim light, rotating gently for ~2 hours. The digestion mixture was sieved sequentially through 250_µm and 38_µm sieves, and the filtrate centrifuged at 700 rpm for 3.5 minutes, discarding the supernatant. The protoplasts were resuspended in wash buffer (0.358_M KCl, 1.0_mM NH_4NO_3, 5.0_mM $CaCl_2 \cdot 2H_2O$, 0.5_mM KH_2PO_4, pH_4.8, ~670 mmol/kg) and pelleted as before. This washing step was repeated. The pellet was resuspended in wash buffer and the protoplasts were counted. Transformation was achieved using a Polyethylene glycol method based on Negrutiu et al. Protoplasts were resuspended at 2 x 10^6/ml in MaMg medium (0.4_M Mannitol, 15 mM $MgCl_2$, 0.1% MES, pH_5.6, ~450_mmol/kg) aliquotting 0.5 ml / treatment (i.e. $1x10^6$ protoplasts / treatment). Samples were heat shocked at 45°C for 5 minutes then cooled to room temperature. 10_µg each of p221.9GRE6 and pMF6HR1PAT (GR) (1_mg/ml) / treatment were added and mixed in gently, followed by immediate addition of 0.5_ml warm (~45°C) PEG solution (40% PEG_3,350 MW in 0.4_M Mannitol, 0.1_M $Ca(NO_3)_2$, pH_8.0), which was mixed in thoroughly but gently. Treatments were incubated at room temperature for 20-25 minutes, then 5_ml 0.292_M KCl (pH_5.6, ~530_mmol/kg) was added step-wise, 1_ml at a time, with mixing. The treatments were incubated for a further

10-15 minutes prior to pelleting the protoplasts by centrifuging as before. Each protoplast treatment was resuspended in 1.5_ml culture medium (MS medium, 2% sucrose, 2_mg/l 2,4-D, 9% Mannitol, pH__5.6, ~700__mmol/kg) +/- 0.0001 M dexamethasone (glucocorticoid). The samples were incubated in 3_cm dishes at 25°C, dark, for 24-48 hours prior to harvesting. Fluorometric assays for GUS activity were performed with the substrate 4-methylumbelliferyl-D-glucuronide using a Perkin-Elmer LS-35 fluorometer (Jefferson et al., 1987). Protein concentrations of tissue homogenates were determined by the Bio-Rad protein assay (Bradford, 1976). The method was repeated for each effector construct.

D. Results

1. Reporter gene assay

A reporter gene construct (p221.9GRE6) was generated containing the GUS reporter gene under the control of a -60 CaMV 35S promoter with 6 copies of the glucocorticoid response element. To test this construct was functional in maize protoplasts a co-transformation assay was performed with the reporter construct p221.9GRE6 and the effector construct pMF6HR1PAT (GR) construct containing the entire glucocorticoid receptor.

Figure 15 shows that Reporter p221.9GRE6 alone or reporter plus effector pMF6HR1PAT (GR) with no activating chemical gave no significant expression. When reporter plus effector were co-transformed into maize protoplasts in the presence of 0.0001 M dexamethasone (glucocorticoid), a significant elevation of marker gene activity was observed (Figure 15). The response is specific to glucocorticoid as the steroid Muristerone A does not lead to induced levels of expression. These studies clearly show the reporter gene construct p221.9GRE6 is capable of monitoring effector /ligand mediated gene expression.

2. Chimeric ecdysone effector constructs mediate inducible expression in maize transient protoplasts assays

A chimeric effector plasmid pMF6GREcRS was constructed, containing the ligand binding domain from the *Drosophila* ecdysone receptor and the DNA binding and transactivation domain from the glucocorticoid receptor. To confirm the reporter gene

construct p221.9GRE6 could respond to a chimeric ecdysone effector construct, a series of co-transformation into maize protoplasts was performed.

Figure 16 shows that reporter (p221.9GRE6) alone or reporter plus effector (pMF6GREcRS) with no activating chemical, gave no significant expression in maize protoplasts. When reporter plus effector were co-transformed into maize protoplasts in the presence of 100_μM Muristerone A, a significant elevation of marker gene activity was observed. The response was specific to Muristerone A, as the steroid dexamethasone did not lead to induced levels of expression. These studies clearly showed the reporter gene construct p221.9GRE6 is capable of monitoring chimeric ecdysone effector /ligand mediated gene expression.

A second chimeric effector construct pMF6GRHEcR, was generated containing the ligand binding domain from *Heliothis* ecdysone receptor. When co-transformed into maize protoplasts with the reporter plasmid p221.9GRE6, no response to 100 μM Muristerone or 100_μM dexamethasone was observed (Figure 17). These data clearly show the *Drosophila* and *Heliothis* ligand binding domains exhibit different properties.

When the effector plasmid pMF6GREcRS, containing the ligand binding domain from *Drosophila*, was tested with the reporter p221.9GRE6 in presence of the non-steroidal ecdysone agonists RH5849 and RH5992 (mimic), no chemical induced reporter gene activity was observed (Figures 18 and 19).

When the effector plasmid pMF6GRHEcR, containing the ligand binding domain from *Heliothis*, was tested with the reporter p221.9GRE6 in presence of the non-steroidal ecdysone agonists RH5992 (mimic), significant chemical induced reporter gene activity was observed (Figure 20). These data demonstrate the ligand binding domain from *Heliothis* has different properties to the *Drosophila* receptor in that the former responded to the non-steroidal ecdysteroid agonist RH5992. Figure 21 demonstrates the effector plasmid pMF6GRHEcR confers RH5992 dependant inducibility on the reporter p221.9GRE6 in a dose responsive manner. Induction was observed in a range from 1_μM-100_μM RH5992.

Example IV - Testing of effector vectors in Tobacco protoplasts

The experiments carried out in the previous example demonstrated the specific effect of RH5992 (mimic) on pMF6GRHEcR in maize protoplasts. It is the aim in this example to show the generic application to plants of the glucocorticoid/*Heliothis* ecdysone

chimeric receptor switch system. Tobacco shoot cultures cv. Samsun, were maintained on solidified MS medium + 3% sucrose in a controlled environment room (16 hour day / 8 hour night at 25°C, 55% R.H), were used as the source material for protoplasts. Leaves were sliced parallel to the mid-rib, discarding any large veins and the slices were placed in CPW13M 13% Mannitol, pH_5.6, ~860_mmol/kg) for ~1 hour to pre-plasmolyse the cells. This solution was replaced with enzyme mixture (0.2% Cellulase R10, 0.05% Macerozyme R10 in CPW9M (CPW13M but 9% Mannitol), pH_5.6, ~600_mmol/kg) and incubated in the dark at 25°C overnight (~16 hours). Following digestion, the tissue was teased apart with forceps and any large undigested pieces were discarded. The enzyme mixture was passed through a 75µm sieve and the filtrate was centrifuged at 600_rpm for 3.5 minutes, discarding the supernatant. The pellet was resuspended in 0.6_M sucrose solution and centrifuged at 600_rpm for 10 minutes. The floating layer of protoplasts was removed using a Pasteur pipette and diluted with CPW9M (pH_5.6, ~560_mmol/kg). The protoplasts were again pelleted by centrifuging at 600_rpm for 3.5 minutes, resuspended in CPW9M and counted. A modified version of the PEG-mediated transformation above was carried out. Protoplasts were resuspended at $2x10^6$/ml in MaMg medium and aliquotted using 200_µl / treatment (i.e. $4x10^5$ protoplasts / treatment). 20_µg each of pMF6GRHEcRS and p221.9GRE6 DNA (1 mg/ml) were added followed by 200_µl PEG solution and the solutions gently mixed. The protoplasts were left to incubate at room temperature for 10 minutes before addition of 5_ml MSP19M medium (MS medium, 3% sucrose, 9% Mannitol, 2_mg/l NAA, 0.5_mg/l BAP, pH_5.6, ~700_mmol/kg) +/- 10 µM RH5992. Following gentle mixing, the protoplasts were cultured in their tubes, lying horizontally at 25°C, light. The protoplasts were harvested for the GUS assay after ~24 hours.

A. Effector construct

(i) Construction of a Dicotyledonous expression vector

The vector produced is a derivative of pMF6. pMF6GREcRS was restriction enzyme digested with PstI to produce 3 fragments namely, 3.4_(Adh Intronless pMF6), 3.2 (GREcRS) and 0.5_(Adh intron I) kb. Isolation and religation of the 3.4 and 3.2 kb fragments resulted in pMF7GREcRS (Figure 22). pMF7GREcRS was restriction enzyme digested with EcoRI/SacI resulting in the 3.4 kb_pMF7 EcoRI/SacI vector which when isolated and purified was ligated to a 1.5 kb EcoRI/XhoI N-terminal end of the

glucocorticoid receptor and the 1.2 kb SalI/SacI *Heliothis* ecdysone C-terminal end sequences to produce pMF7GRHEcR (Figure 23).

B. Reporter plasmid

The reporter plasmids constructed for the maize transient experiments were the same as those used without alteration in the tobacco leaf protoplast transient expression experiments.

C. Results - Chimeric ecdysone effector constructs mediate inducible expression in tobacco transient protoplast assays

Experiments were performed to demonstrate that the effector plasmid pMF6GRHEcR can confer chemical dependant inducible expression on the reporter p221.9GRE6 in tobacco mesophyll protoplasts.

Figure 24 shows that reporter (p221.9GRE6) alone or reporter plus effector (pMF7GRHEcR) with no activating chemical, gave no significant expression in tobacco protoplasts. When reporter plus effector were co-transformed into tobacco protoplasts in the presence of 10_μM RH5992, a significant elevation of marker gene activity was observed. These data show a chimeric ecdysone effector construct, containing the *Heliothis* ligand binding domain can confer non-steroidal ecdysteroid dependant expression on reporter gene constructs in both monocotyledonous and dicotyledonous species.

Example V - Chimeric activity in Mammalian cells

A. Effector constructs

(i) Construction of Glucocorticoid/*Heliothis* ecdysone chimeric receptor.

The mammalian expression vector used in this experiment was pcDNA3 (Invitrogen). The GRHEcR 2.7_kb BamHI DNA fragment (isolated from pMF6GRHEcR) was introduced into the pcDNA3 BamHI vector. The recombinants were oriented by restriction enzyme mapping. The DNA sequence of the junctions was determined to ensure correct orientation and insertion (pcDNA3GRHEcR, Figure 25).

B. Reporter construct

The reporter plasmid for mammalian cell system was produced by taking pSWBGAL plasmid and replacing the CRESW SpeI/ClaI fragment for a synthetic 105 bp DNA fragment containing 4 copies of the glucocorticoid response element (GRE) and flanked by SpeI at the 5' end and AflII at the 3' end.

The oligonucleotides were synthesised using the sequences:

GREspeI

5'ctagttgtacaggatgttctagctactcgagtagctagaacatcctgtacagtcgagtagctagaacatcctgtacagtcgagtag ctagaacatcctgtacac 3' **(SEQ ID NO: 44)**;

GREafl2

5'ttaagtgtacaggatgttctagctactcgactgtacaggatgttctagctactcgactgtacaggatgttctagctactcgagtagct agaacatcctgtacaa 3' **(SEQ ID NO: 45)**.

The two oligonucleotides were purified annealed and ligated to pSWBGAL SpeI/AflII to produce pSWGRE4 (Figure 26).

C. Results - Chimeric HEcR drives transient reporter gene expression in mammalian cells

No expression was detected when a reporter gene construct pSWGRE4, comprising of a minimal β-globin promoter containing four copies of the glucocorticoid response element, fused to a β-galactosidase reporter gene, was introduced into CHO cells. Similarly, no expression was detected when pSWGRE4 and an effector plasmid pCDNA3GRHEcR, containing the transactivation and DNA binding domain from the glucocorticoid receptor and the ligand binding domain from the *Heliothis* ecdysone receptor, under the control of the CMV promoter were co-transformed into CHO-K1 or HEK293 cells. When co-transformed CHO (Figure 27) and HEK293 cells (Figure 28) were incubated in the presence of the non-steroidal ecdysone agonists RH5992 (mimic), significant chemical induced reporter gene activity was observed. Equally, induction of reporter activity was observed when HEK293 cells transfected with pcDNA3GRHEcR and reporter were treated with Muristerone A (Figure 28).

Example VI - Screening system allows new chemical activators and modified ligand binding domains to be tested in Mammalian cells

The basis of a screening system ~~are~~ **is** in place after the demonstration that the chimeric receptor was activated in the presence of RH5992. A screen was carried out

using CHO cells transiently transfected with both pSWGRE4 (reporter) and pcDNA3GRHEcR (effector) constructs. In the first instance 20 derivatives compounds of RH5992 were screened. It was observed that 7 out of the 20 compounds gave an increased reporter gene activity compared to untreated cells. A second screen was carried out in which 1000 randomly selected compounds were applied to transiently transfected CHO cells. Two compounds were found to activate reporter gene activity above that from the untreated controls. The second screen suggest that this cell based assay is a robust and rapid way to screen a small library of compounds, where a thousand compounds can be put through per week.

Example ~~V~~ VII - Stably transformed Tobacco plants

A. Stable Tobacco vectors

The components of the stable Tobacco vectors were put together in pBluescript prior to transfer into the binary vector. The production of stable transformed plants entails the production of a vector in which both components of the switch system (i.e. effector and reporter) are placed in the same construct to then introduce into plants.

The methodology described below was used to produce four different stable Tobacco vectors. The method involves three steps:

1. pBluescript SK HindIII/EcoRI vector was ligated to either GRE6-4635SCaMVGUSNOS HindIII/EcoRI (from p221.10GRE6) or GRE6-6035SCaMVGUSNOS HindIII/EcoRI (from p221.9GRE6) resulting in plasmid pSK-46 and pSK-60.
2. This step involves the addition of the chimeric receptor (35SGRHEcRNOS or 35SGRVP16HEcRNOS) to pSK-60 or pSK-46. Thus a pSK-60 (or pSK-46) XbaI vector was ligated with either the 3.4 kb 35SGRHEcRNOS XbaI or the 3.0 kb 35SGRVP16HEcRNOS XbaI DNA fragment to produce pSKES1 (pSKGRE6-6035SCaMVGUSNOS-35SGRHEcRNOS), pSKES2 (pSKGRE6-4635SCaMVGUSNOS-35SGRHEcRNOS), pSKES3 (pSKGRE6-6035SCaMVGUSNOS-35SGRVP16HEcRNOS) and pSKES4 (pSKGRE6-4635SCaMVGUSNOS-35SGRVP16HEcRNOS).

3. Transfer from pBluescript based vectors to binary vectors. The transfer of the ES1 (Figure 29) ES2 (Figure 30), ES3 (Figure 31) or ES4 (Figure 32) DNA fragments into the binary vector JR1 involves five steps:

(i) Restriction enzyme digestion of pSKES1 (ES2, ES3, and ES4) with ApaI and NotI to liberate the insert from the vector pBluescript.

(ii) The two DNA fragments were BamHI methylated for 2 hours at 37°C in TRIS-HCl, MgCl, 80~~uM~~ **µM** SAM (S-adenosylmethionine) and 20 U of BamHI methylase.

(iii) Ligate an ApaI/NotI linker onto the fragment. The linker was designed to have an internal BamHI site:
ApaBNot1 5' cattggatccttagc 3' **(SEQ ID NO: 46)** and
ApaBNot2 5' ggccgctaaggatccaatgggcc 3' **(SEQ ID NO: 47)**.

(iv) Restriction enzyme digest the protected and linkered fragment with BamHI and fractionate the products on a 1% (w/v) ~~Agarose~~ **agarose** gel. The protected DNA fragment (5.5 kb) was cut out of the gel and purified.

(v) A ligation of JRI BamHI vector with the protected band was carried out to produce JRIESI (JRIES2, JRIES3 or JRIES4). The DNA of the recombinant was characterised by restriction mapping and the sequence of the junctions determined.

The plant transformation construct pES1, containing a chimeric ecdysone receptor and a reporter gene cassette, was transferred into Agrobacterium tumefaciens LBA4404 using the freeze/thaw method described by Holsters et al. (1978). Tobacco (*Nicotiana tabacum* cv *Samsun*) transformants were produced by the leaf disc method (Bevan, 1984). Shoots were regenerated on medium containing 100 mg/l kanamycin. After rooting, plantlets were transferred to the glasshouse and grown under 16 hour light/ 8 hour dark conditions.

B. Results - Chimeric ecdysone effector constructs mediate inducible expression in stably tobacco plants

Transgenic tobacco plants were treated in cell culture by adding 100 µM RH5992 to MS media. In addition seedlings were grown hydroponically in the presence or absence of RH5992. In further experiments 5 mM RH5992 was applied in a foliar application to 8 week old glasshouse grown tobacco plants. In the three methods described uninduced

levels of GUS activity were comparable to a wild type control, while RH5992 levels were significantly elevated.

Ecdysone switch modulation and optimisation

Example VIII - Yeast indicator strains for primary screen of chemical libraries

A set of yeast indicator strains was produced to use as a primary screen to find chemicals which may be used in the gene switch. The properties of the desired chemicals should include high affinity resulting in high activation but with different physico-chemical characteristics so as to increase the scope of application of the technology. Moreover, the production of this strain also demonstrates the generic features of this switch system.

A. Effector vector

A base vector for yeast YCp15Gal-TEV-112 was generated containing:

Backbone - a modified version of pRS315 (Sikorski and Hieter (1989) Genetics 122, 19-27)- a shuttle vector with the LEU2 selectable marker for use in yeast;

ADH1 promoter (BamHI- Hind III fragment) and ADH1 terminator (Not I- Bam HI fragment) from pADNS (Colicelli et al PNAS 86, 3599-3603);

DNA binding domain of GAL4 (amino acids 1-147; GAL4 sequence is Laughon and Gesteland 91984) Mol. Cell Biol. 4, 260-267) from pSG424 (Sadowski and Ptashne (1989) Nuc. Acids Res. 17, 7539);

Activation domain - an acidic activation region corresponding to amino acids 1-107 of activation region B112 obtained from plasmid pB112 (Ruden et al (1991) Nature 350, 250-252).

The plasmid contains unique Eco RI, Nco I and Xba I sites between the DNA binding domain and activation domains.

Into this vector a PCR DNA fragment of the *Heliothis* ecdysone receptor containing the hinge, ligand binding domains and the C-terminal end was inserted. The 5' oligonucleotide is flanked by an NcoI restriction recognition site and begins at amino acid 259: HecrNcoI 5' aattccatggtacgacgacagtagacgatcac 3' **(SEQ ID NO: 48)**.

The 3' oligonucleotide is flanked by an XbaI site and encodes for up to amino acid 571: HecRXbaI 5' ctgaggtctagagacggtggcgggcggcc 3' **(SEQ ID NO: 49)**.

The PCR was carried out using vent polymerase with the conditions described in Example IA. The fragment was restriction enzyme digested with NcoI and XbaI purified and ligated into YCp15GALTEV112 NcoI/XbaI vector to produce YGALHeCRB112 or TEV-B112 (Figure 34). In order to reduce constitutive activity of the YGALHeCRB112 plasmid a YGALHeCR plasmid was produced in which the B112 activator was deleted by restriction enzyme digesting YGALHeCRB112 with XbaI/SpeI followed by ligation of the resulting vector (i.e. SpeI and XbaI sites when digested produce compatible ends) (TEV-8, Figure 33). An effector plasmid was constructed whereby the B112 transactivating domain was excised from YGalHecRB112 with XbaI and replaced with the VP16 transactivation domain DNA fragment (encoding amino acids 411 and 490 including the stop codon). The resulting vector was named YGalHecRVP16 or TEVVP16-3 (Figure 35).

B. Reporter construction for yeast

The *S. cerevisiae* strain GGY1::171 (Gill and Ptashne (1987) Cell 51, 121-126), YT6::171 (Himmelfarb et al (1990) Cell 63, 1299-1309) both contain reporter plasmids consisting of the GAL4-responsive GAL1 promoter driving the *E. coli* β-galactosidase gene. These plasmids are integrated at the URA3 locus. The reporter strain YT6::185 contains the reporter plasmid pJP185 (two synthetic GAL4 sites driving the B-galactosidase gene) integrated at the URA3 locus of YT6 (Himmelfarb et al). (Note- the parental strains YT6 and GGY1 have mutations in the GAL4 and GAL80 genes, so the reporter genes are inactive in the absence of any plasmids expressing GAL4 fusions).

C. Yeast assay

Standard transformation protocols (Lithium acetate procedure) and selection of colonies by growth of cells on selective media (leucine minus medium in the case of the YCp15Gal-TEV-112 plasmid)- as described in Guthrie and Fink)(1991) Guide to Yeast Genetics and Molecular Biology. Methods in Enzymology Vol. 194 Academic Press and the reporter gene assay is a modification of that described in ~~Ausabel~~ **Ausubel** et al., (1993) Current Protocols in Molecular Biology (Wiley) Chapter 13).

D. Results - Automated screening system allows new chemical activators and modified ligand binding domains to be tested in yeast

An effector vector pYGALHEcRB112 has been generated containing a GAL4 DNA binding domain, a B112 activation domain and the ligand binding region from *Heliothis virescens*. In combination with a GAL reporter vector, pYGALHEcRB112 form the basis of a rapid, high throughput assay which is cheap to run. This cell-based assay in yeast (*Saccharomyces cerevisiae*) will be used to screen for novel non-steroidal ecdysone agonists which may of commercial interest as novel insecticides or potent activators of the ecdysone gene switch system. The demonstration of an efficient system to control gene expression in a chemical dependant manner, forms the basis of an inducible system for peptide production in yeast.

The yeast screening system forms the basis of a screen for enhanced ligand binding using the lac Z reporter gene vector to quantitatively assay the contribution of mutation in the ligand binding domain. Alternatively, enhanced ligand binding capabilities or with a selection cassette where the lac Z reporter is replaced with a selectable marker such as uracil (URA 3), tryptophan (Trp1) or leucine (Leu2), and histidine (His). Constructs based on pYGALHEcRB112 with alterations in the ligand binding domain are grown under selection conditions which impair growth of yeast containing the wild type ligand binding domain. Those surviving in the presence of inducer are retested and then sequenced to identify the mutation conferring resistance.

Example IX - Optimisation of chimeric receptor using a strong transactivator

A. Construction of mammalian expression plasmid with chimeric receptor containing ~~Herpex~~ **Herpes** Simplex VP16 protein sequences.

The construction of this chimeric receptor is based on replacing the sequences encoding for the glucocorticoid receptor transactivating domain with those belonging to the VP16 protein of ~~Herpex~~ **Herpes** simplex. Thus PCR was used to generate three fragments all to be assembled to produce the chimeric receptor. The PCRs were carried out as described in Example II, iii. The first fragment includes the Kozak sequences and methionine start site of the glucocorticoid receptor to amino acid 152 of the glucocorticoid receptor. The oligonucleotides used for the generation of this fragment included an EcoRI site at the 5' end: GR1A 5' atatgaattccaccatggactccaaagaatc 3' **(SEQ ID NO: 50)**, and at

the 3' end a NheI restriction enzyme recognition site: GR1B 5' atatgctagctgtgggggcagcagacacagcagtgg 3' **(SEQ ID NO: 51)**.

The second fragment also belongs to the glucocorticoid receptor and begins with a NheI site in frame with amino acid 406: GR2A 5' atatgctagctccagctcctcaacagcaacaac 3' **(SEQ ID NO: 52)**, and ends with a XhoI site at amino acid 500: GR2B 5' atatctcgagcaattccttttatttttttc 3' **(SEQ ID NO: 53)**.

The two fragments were introduced into pSKEcoRI/SacI in a ligation containing GR1A/B EcoRI/NheI, GR2A/B NheI/XhoI and HEcR SalI/SacI (from pSKHEcRDEF) to yield pSKGRDHEcR. The GR sequences and junctions of the ligation were found to be mutation free.

The third fragment to be amplified was a sequence between amino acid 411 to 490 of the herpes simplex VP16 protein. The amplified fragment was flanked with SpeI recognition sites. SpeI produces compatible ends to those of NheI sites. The oligonucleotides used: VP16C 5' attactagttctgcggccccccgaccgat 3' **(SEQ ID NO: 54)** and VP16E 5' aattactagtcccaccgtactcgtcaattcc 3' **(SEQ ID NO: 55)** produced a 180 bp fragment which was restriction enzyme digested with SpeI and introduced into pSKGRΔHEcR NheI vector to produce pSKGRVP16HEcR. The DNA from the latter was sequenced and ~~and~~ found to be mutation free, the junctions were also shown to be in frame with those of the glucocorticoid receptor.

The 2.2 kb EcoRV/NotI GRVP16HEcR fragment was introduced into a pcDNA3 EcoRV/NotI vector resulting in pcDNA3GRVP16HEcR (Figure 36).

B. Construction of plant transient expression effector plasmids containing the chimeric receptor with VP16 sequences

The same procedure was carried out to clone the GRVP16HeCR DNA fragment into tobacco(pMF7b) and maize (pMF6) expression vectors. A 2.2_kb BamHI DNA fragment was isolated from pcDNA3GRVP16HeCR and ligated in to the pMF6 BamHI (or pMF7b BamHI) vector to produce pMF6GRVP16HeCR (Figure 37) (or pMF7GRVP16HeCR) (Figure 38).

C. Results - Addition of strong activation domains enhance ecdysone switch system

The VP16 transactivation domain from herpes simplex virus has been added to a maize protoplast vector pMF6GRHEcR to generate the vector pMF6GRVP16HEcR.

When co-transformed into maize protoplasts with the reporter construct p221.9GRE6, in the presence of 100μM RH5992, enhanced levels of expression were seen over pMF6GRHEcR. Figure 39, shows that RH5992 is able to induce GUS levels comparable to those observed with the positive control (p35SCaMVGUS), moreover, a dose response effect is observable.

VP16 enhanced vectors (pES3 and pES4) have been generated for stable transformation of tobacco. Following transformation transgenic progeny containing pES3 and pES4, gave elevated GUS levels following treatment with RH5992, relative to comparable transgenic plants containing the non-VP16 enhanced vectors pES1 and pES2.

An enhanced mammalian vector pcDNA3GRVP16HEcR was prepared for transient transfection of mammalian cell lines. Elevated reporter gene activities were obtained relative to the effector construct (pCDNA3GRHEcR) without the VP16 addition.

"Acidic" activation domains are apparently "universal" activators in eukaryotes (Ptashne (1988) Nature 335 683-689). Other suitable acidic activation domains which have been used in fusions are the activator regions of GAL4 itself (region I and region II; Ma and Ptashne (Cell (1987) 48, 847-853), the yeast activator GCN4 (Hope and Struhl (1986) Cell 46, 885-894) and the herpes simplex virus VP16 protein (Triezenberg et al (1988) Genes Dev. 2, 718-729 and 730-742).

Other acidic and non-acidic transcriptional enhancer sequences for example from plant fungal and mammalian species can be added to the chimeric ecdysone receptor to enhance induced levels of gene expression.

Chimeric or synthetic activation domains can be generated to enhance induced levels of gene expression.

Example X - Optimisation by replacement of *Heliothis* ligand binding domain in chimeric effector for that of an ecdysone ligand binding domain of another species

Mutagenesis of the ecdysone ligand binding domain results in the increased sensitivity of the chimeric receptor for activating chemical. This can be achieved by deletions in the ligand binding domain, use of error prone PCR (Caldwell et al., PCR Meth. Applic 2, 28-33 1992), and in vitro DNA shuffling PCR (Stemmer, Nature 370, 389-391 1994). To enhance the efficacy of the listed techniques we have developed a screening system for enhanced levels of induced expression (see below).

An alternative strategy to the mutation of a known ligand binding domain is to identify insect species which are particularly sensitive to ecdysteroid agonists. For example *Spodoptera exigua* is particularly sensitive to RH5992. To investigate the role of the ecdysone receptor ligand binding domain in increased sensitivity to RH5992 we have isolated corresponding DNA sequences from of *S. exigua* (Figure 40, ~~Sequence ID No.~~ **SEQ ID NO:** 6). Figure 41, ~~Sequence ID No. 7~~ shows a protein alignment of the hinge and ligand binding domains of the *Heliothis virescens* **(SEQ ID NO: 5)** and *Spodoptera exigua* **(SEQ ID NO: 7)** ecdysone receptors. The protein sequence between the two species is well conserved.

Results - Manipulation of the ligand binding domain leads to enhanced induced expression

Isolation of an ecdysone ligand binding domain from another lepidopteran species was carried out by using degenerate oligonucleotides and PCR of first strand cDNA (Perkin Elmer, cDNA synthesis Kit) of the chosen species. The degenerate oligonucleotides at the 5' end were HingxhoA and B and at the 3' end ligandxA/B

```
HingxhoA 5' attgctcgagaaagiccigagtgcgtigticc 3'(SEQ ID NO: 56)
                                  a  t

HingxhoB 5' attgctcgagaacgiccigagtgtgtigticc 3'(SEQ ID NO: 57)
                                  a  c

LigandxA 5' ttactcgagiacgtcccaiatctcttciaggaa 3'(SEQ ID NO: 58)
                           a           c

ligandxB 5' ttactcgagiacgtcccaiatctcctciaagaa 3'(SEQ ID NO: 59)
                           a           t
```

RNA was extracted from 4th instar larvae of *Spodoptera exigua* since *Spodoptera exigua* appears to be more sensitive to RH5992 than *Heliothis* (Smagghe and Degheele, 1994). The first strand cDNA was used in PCR reactions under the following conditions 20 mM Tris-HCL (pH 8.4), 50 mM KCl, 1.5 mM $MgCl_2$, 200 mM dNTPs (dATP, dCTP, dGTP and dTTP) and 0.02 U/ml *Taq* DNA polymerase and in the presence of 1 µg of each Hinge (5' 3') and Ligand (5'3') oligonucleotides. The PCR cycling conditions were 94°C for 1 minute, 60°C for 2 minutes, and 72°C for 1 minute and 35 cycles were carried out. A sample of the completed reaction was fractionated in a 1% agarose (w/v) 1 x TBE gel, and the resulting 900 bp fragment was subcloned into pCRII vector (Invitrogen). The resulting clone (pSKSEcR 1-10) were further characterised and sequenced.

Example ~~X~~ XI -Manipulation of reporter gene promoter regions can modulate chemical induced expression

The context of the effector response element in the reporter gene promoter can be used to modulate the basal and induced levels of gene expression. Six copies of the glucocorticoid response element were fused to 46 bp or 60 bp of the CaMV 35S promoter sequence. When used with the effector construct pMF7GRHEcRS the reporter gene construct containing 46 bp of the CaMV 35S promoter gave reduced basal and induced levels of GUS expression relative to the 60 bp reporter construct (Figure 42).

Constructs for plant transformation (pES1 and ES2) have been generated to demonstrate the size of minimal promoter can be used to modulate the basal and induced levels of gene expression in plants.

The number and spacing of response elements in the reporter gene promoter can be adjusted to enhance induced levels of trans-gene expression.

The utility of a two component system (effector and reporter gene cassettes) allows the spatial control of induced expression. Trans-gene expression can be regulated in an tissue specific, organ specific or developmentally controlled manner. This can be achieved by driving the effector construct from a spatially or temporally regulated promoter.

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